PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
BO 41497		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NL 98/00186	03/04/1998	
Applicant		
STICHTING VOOR DE TECHNISCHE WETENSCHAPPEN et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [X] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:
   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [X] None of the figures.

INTERNATIONAL SEARCH REPORT

.ernational application No.
PCT/NL 98/00186

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2,7
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Subclaims 2 and 7 revealed to be not searchable since they are unclear and lack clarity (claim 2) and comprise no technical features suitable to perform any search (claim 7).

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 98/00186

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 31569 A (NEDERLANDEN STAAT ;EMBDEN JOHANNES DIRK ANTHONIE (NL); SCHOULS LEE) 23 November 1995 cited in the application see the whole document ---	1,3-6, 10-14, 17-25
X	KAMERBEEK J ET AL.: "Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 35, no. 4, 1997, pages 907-914, XP002091620 cited in the application see the whole document --- -/--	1,3-6, 10-14, 17-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 1999	16/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Knehr, M

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GROENEN P M ET AL.: "Nature of DNA polymorphism in the direct repeat cluster of Mycobacterium tuberculosis; Application for strain differentiation by a novel typing method" MOLECULAR MICROBIOLOGY, vol. 10, no. 5, 1993, pages 1057-1065, XP002091621 cited in the application see the whole document	1,3-6, 10-14, 17-25
X	SOOLINGEN VAN D ET AL: "USE OF VARIOUS GENETIC MARKERS IN DIFFERENTIATION OF MYCOBACTERIUM BOVIS STRAINS FROM ANIMALS AND HUMANS AND FOR STUDYING EPIDEMIOLOGY OF BOVINE TUBERCULOSIS" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 32, no. 10, October 1994, pages 2425-2433, XP000647581	17-19
Y	see the whole document	8-12,15, 16
X	US 5 691 136 A (KOEUTH THEARITH ET AL) 25 November 1997	1,8-12, 15-19
Y	see the whole document	20-22
Y	KLENK H-P ET AL.: "The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon Archaeoglobus fulgidus" NATURE, vol. 390, 1997, pages 364-370, XP002091622 cited in the application see abstract; table 1	20-22
Y	MOJICA F J M ET AL.: "Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferx volcanii and could be involved in replicon partitioning" MOLECULAR MICROBIOLOGY, vol. 17, no. 1, 1995, pages 85-93, XP002091623 cited in the application see abstract see page 85, column 2, paragraph 1 - page 87, column 1, paragraph 2; figure 1	1,3-6

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/NL 98/00186

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropnate, of the relevant passages	Relevant to claim No.
Y	LIEBL W ET AL.: "Analysis of a Thermotoga maritima DNA fragment encoding two similar thermostable cellulases, CelA and CelB, and characterization of the recombinant enzymes" MICROBIOLOGY, vol. 142, 1996, pages 2533-2542, XP002091624 see abstract see page 2536, column 1, paragraph 3 - page 2537, column 1, paragraph 1; figures 3,4	1,3-6
Y	SHANGKUAN Y-H ET AL.: "Diversity of DNA sequences among Vibrio cholerae O1 and non-O1 isolates detected by whole-cell repetitive element sequence-based polymerase chain reaction" JOURNAL OF APPLIED MICROBIOLOGY, vol. 82, no. 3, 1997, pages 335-344, XP002091625 see the whole document	1,3-6, 8-12,15, 16
A	SOOLINGEN VAN D ET AL: "COMPARISON OF VARIOUS REPETITIVE DNA ELEMENTS AS GENETIC MARKERS FOR STRAIN DIFFERENTIATION AND EPIDEMIOLOGY OF MYCOBACTERIUM TUBERCULOSIS" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 31, no. 8, August 1993, pages 1987-1995, XP000647582 cited in the application	
A	VERSALOVIC J ET AL.: "Distribution of repetitive DNA sequences in eubacteria and application to fingerpriting of bacterial genomes" NUCLEIC ACIDS RESEARCH, vol. 19, no. 24, 1991, pages 6823-6831, XP002091626 see the whole document	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/NL 98/00186

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9531569 A	23-11-1995	AU 690118 B	23-04-1998
		AU 6858294 A	05-12-1995
		EP 0760005 A	05-03-1997
		JP 10500011 T	06-01-1998
US 5691136 A	25-11-1997	AU 2931692 A	21-05-1993
		CA 2121696 A	29-04-1993
		EP 0610396 A	17-08-1994
		WO 9308297 A	29-04-1993
		US 5523217 A	04-06-1996

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 01 December 1999 (01.12.99)	
International application No. PCT/NL98/00186	**Applicant's or agent's file reference** BO 41497
International filing date (day/month/year) 03 April 1998 (03.04.98)	**Priority date** (day/month/year)
Applicant VAN EMBDEN, Johannes, Dirk, Anthonie et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

02 November 1999 (02.11.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer A. Karkachi
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

REC'D 07 JUL 2000

WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
BO 41497		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL98/00186	03/04/1998	03/04/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant STICHTING VOOR DE TECHNISCHE WETENSCHAPPEN et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/11/1999	04.07.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Bradbrook, D Telephone No. +49 89 2399 7413

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL98/00186

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-21	as originally filed			

Claims, No.:

1-25	as received on	02/06/2000	with letter of	02/06/2000

Drawings, No.:

1-5	as originally filed			
6	as received on	15/06/2000	with letter of	15/06/2000

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 2,7.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 2,7.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1,3-6,8-25
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1,3-6,8-25
Industrial applicability (IA)	Yes:	Claims	1,3-6,8-25
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Section I

1. Sequence listing pages 1-6, filed with the letter of 19.01.99, do not form part of the application (Rule 13ter.1(f) PCT). It is noted that new Fig.6, filed with the letter of 15.06.00, lists 18 sequences, whereas the aforementioned sequence listing has only 17 sequences. Sequences 17 and 18 in Fig.6 arise from previously filed SEQ ID NO:17, in which the direct repeats from two different bacteria were mistakenly joined to form one sequence (cf Table II, p.14: Thermoautotrophicum and Archaeoglobus fulgidis). Therefore, no new subject-matter has been added. However, the present sequence listing is consequently incorrect.

Section V

2. The applicant's observations submitted with the amended claims have been considered in establishing this report.

3. Reference is made to the following documents:

   D1: WO-A-95 31569 (The State of Netherlands; 23.11.95);
   D2: Klenk et al., Nature, Vol.390, pp.364-370 (1997);
   D3: Mojica et al., Molecular Microbiology, Vol.17, pp.85-93 (1995);
   D4: Liebl et al., Microbiology, Vol.142, p.2533-2542 (1996).

4. Novelty and Inventive step (Article 33(2) and (3) PCT)

a. The present application concerns a method of in vitro amplification of nucleic acid, and its use in methods of detecting and identifying bacteria in a sample. A pair of primers is used in the amplification, which primers are complementary to the direct repeat sequence of a bacterium other than one belonging to the M. tuberculosis complex. The direct repeats, of length 20-50 base pairs, occur 5-60 times in a region of the bacterial genome, and are separated by spacer sequences which are 20-50 base pairs long and non-repetitive. The identification of the bacteria is according to the spacer sequences. Also claimed are primer pairs and a kit.

b. The method of amplification defined by claim 1 appears to be novel: the closest

prior art is considered to be D1, in which the same method is used, but is applied only to microbes of the M. tuberculosis complex (see D1: abstract, claims and p.5, l.24-35).

c. The difference between claim 1 and D1 is that the method is applied to different bacteria. Such an analogous use of a known method is usually not accepted as involving an inventive step. In the present case, the inventiveness appears to rely on the unexpected occurrence of direct variant repeats (DVRs) in bacteria other than those of the M. tuberculosis complex (see present description, p.4 l.26-p.5 l.11). However, such DVRs are known in E. coli (description: p.8, l.26-28), as well as in other bacteria such as Archaeoglobus fulgidus (D2: Table 1 and p.366, col.1, "Repetitive elements"), Haloferax sp. (D3: Summary and Fig.1), and Thermotoga maritima (D4: Fig.3 and paragraph bridging pages 2536 and 2537).

As pointed out in the present application (p.2, l.2-9), the problems in differentiating M. tuberculosis bacteria are the same for other bacteria, for which quicker and simpler methods are required. In the knowledge that DVRs are not confined to M. tuberculosis complex microbes, it would be desirable and straightforward for the skilled person to analyse other bacterial sequences, freely available from databases, to identify DVR structures in a wide range of bacteria. The screen in D1 (p.13, l.5-15) did not detect bacteria other than from the M. tuberculosis complex; this is to be expected, as the direct repeats are heterogeneous (compare those in D1-D4). However, a screen using each identified direct repeat would be expected to enable bacteria to be grouped according to those having the same repeat. Thus, as is the case with M. tuberculosis, any one direct repeat would be the basis for identifying a group of bacterial species or strains which would be distinguishable within the group by the interspersed non-similar sequences.

Therefore, claim 1 and dependent claims 3-6 and 8-11 are considered to be not inventive.

d. As with claim 1, the methods of claims 12 and 17 were applied to the M. tuberculosis complex in D1 (cf D1 claims 7-14). Therefore, present claims 12-19 appear to be not inventive.

e. The primer pairs of claims 20-23 correspond essentially to known direct repeat sequences (e.g. of E. coli and A. fulgidus: see Table II) and as such cannot be considered inventive in the light of the foregoing comments (also cf D1: claims 18 and 19).

f. Similarly, the kits of claims 24 and 25 are not inventive (cf D1: claim 21).

Section VIII

5. Deficiencies under Article 6 PCT are as follows;

a. Terms such as "in particular" (claims 20 and 22) have no limiting effect on the scope of the claims in which they are used, so that any feature following such expressions is considered to be entirely optional (PCT Guidelines C-III 4.6).

b. In claims 21 and 23, the direct repeats are "present in the Direct Region of SEQ ID No. [2][8]"; this is unclear, as the said direct repeats have their sequences defined by the whole of SEQ ID No. 2 or 8.

c. Claim 9 is unclear, as it lists Staphylococcus and Streptococcus, which are Gram positive cocci, yet refers back to claim 8, which concerns only Gram negative bacteria. The same applies to claim 16 in referring to claim 15.

d. Claim 20 is rendered unclear by the incorrect sequence listing (see Section I).

e. SEQ ID NO:2 of Fig.6 appears to be incorrect, as it is one nucleotide shorter than its equivalent in the sequence listing and in Table II of the description. This renders claims 20-22 unclear.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
BO 41497		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL98/00186	03/04/1998	03/04/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant STICHTING VOOR DE TECHNISCHE WETENSCHAPPEN et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/11/1999	04.07.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Bradbrook, D Telephone No. +49 89 2399 7413

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL98/00186

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-21	as originally filed			

Claims, No.:

1-25	as received on	02/06/2000	with letter of	02/06/2000

Drawings, No.:

1-5	as originally filed			
6	as received on	15/06/2000	with letter of	15/06/2000

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL98/00186

☒ claims Nos. 2,7.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 2,7.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1,3-6,8-25
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1,3-6,8-25
Industrial applicability (IA)	Yes:	Claims	1,3-6,8-25
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/NL98/00186

Section I

1. Sequence listing pages 1-6, filed with the letter of 19.01.99, do not form part of the application (Rule 13ter.1(f) PCT). It is noted that new Fig.6, filed with the letter of 15.06.00, lists 18 sequences, whereas the aforementioned sequence listing has only 17 sequences. Sequences 17 and 18 in Fig.6 arise from previously filed SEQ ID NO:17, in which the direct repeats from two different bacteria were mistakenly joined to form one sequence (cf Table II, p.14: Thermoautotrophicum and Archaeoglobus fulgidis). Therefore, no new subject-matter has been added. However, the present sequence listing is consequently incorrect.

Section V

2. The applicant's observations submitted with the amended claims have been considered in establishing this report.

3. Reference is made to the following documents:

   D1: WO-A-95 31569 (The State of Netherlands; 23.11.95);
   D2: Klenk et al., Nature, Vol.390, pp.364-370 (1997);
   D3: Mojica et al., Molecular Microbiology, Vol.17, pp.85-93 (1995);
   D4: Liebl et al., Microbiology, Vol.142, p.2533-2542 (1996).

4. Novelty and Inventive step (Article 33(2) and (3) PCT)

a. The present application concerns a method of in vitro amplification of nucleic acid, and its use in methods of detecting and identifying bacteria in a sample. A pair of primers is used in the amplification, which primers are complementary to the direct repeat sequence of a bacterium other than one belonging to the M. tuberculosis complex. The direct repeats, of length 20-50 base pairs, occur 5-60 times in a region of the bacterial genome, and are separated by spacer sequences which are 20-50 base pairs long and non-repetitive. The identification of the bacteria is according to the spacer sequences. Also claimed are primer pairs and a kit.

b. The method of amplification defined by claim 1 appears to be novel: the closest

prior art is considered to be D1, in which the same method is used, but is applied only to microbes of the M. tuberculosis complex (see D1: abstract, claims and p.5, l.24-35).

c. The difference between claim 1 and D1 is that the method is applied to different bacteria. Such an analogous use of a known method is usually not accepted as involving an inventive step. In the present case, the inventiveness appears to rely on the unexpected occurrence of direct variant repeats (DVRs) in bacteria other than those of the M. tuberculosis complex (see present description, p.4 l.26-p.5 l.11). However, such DVRs are known in E. coli (description: p.8, l.26-28), as well as in other bacteria such as Archaeoglobus fulgidus (D2: Table 1 and p.366, col.1, "Repetitive elements"), Haloferax sp. (D3: Summary and Fig.1), and Thermotoga maritima (D4: Fig.3 and paragraph bridging pages 2536 and 2537).

As pointed out in the present application (p.2, l.2-9), the problems in differentiating M. tuberculosis bacteria are the same for other bacteria, for which quicker and simpler methods are required. In the knowledge that DVRs are not confined to M. tuberculosis complex microbes, it would be desirable and straightforward for the skilled person to analyse other bacterial sequences, freely available from databases, to identify DVR structures in a wide range of bacteria. The screen in D1 (p.13, l.5-15) did not detect bacteria other than from the M. tuberculosis complex; this is to be expected, as the direct repeats are heterogeneous (compare those in D1-D4). However, a screen using each identified direct repeat would be expected to enable bacteria to be grouped according to those having the same repeat. Thus, as is the case with M. tuberculosis, any one direct repeat would be the basis for identifying a group of bacterial species or strains which would be distinguishable within the group by the interspersed non-similar sequences.

Therefore, claim 1 and dependent claims 3-6 and 8-11 are considered to be not inventive.

d. As with claim 1, the methods of claims 12 and 17 were applied to the M. tuberculosis complex in D1 (cf D1 claims 7-14). Therefore, present claims 12-19 appear to be not inventive.

e. The primer pairs of claims 20-23 correspond essentially to known direct repeat sequences (e.g. of E. coli and A. fulgidus: see Table II) and as such cannot be considered inventive in the light of the foregoing comments (also cf D1: claims 18 and 19).

f. Similarly, the kits of claims 24 and 25 are not inventive (cf D1: claim 21).

Section VIII

5. Deficiencies under Article 6 PCT are as follows;

a. Terms such as "in particular" (claims 20 and 22) have no limiting effect on the scope of the claims in which they are used, so that any feature following such expressions is considered to be entirely optional (PCT Guidelines C-III 4.6).

b. In claims 21 and 23, the direct repeats are "present in the Direct Region of SEQ ID No. [2][8]"; this is unclear, as the said direct repeats have their sequences defined by the whole of SEQ ID No. 2 or 8.

c. Claim 9 is unclear, as it lists Staphylococcus and Streptococcus, which are Gram positive cocci, yet refers back to claim 8, which concerns only Gram negative bacteria. The same applies to claim 16 in referring to claim 15.

d. Claim 20 is rendered unclear by the incorrect sequence listing (see Section I).

e. SEQ ID NO:2 of Fig.6 appears to be incorrect, as it is one nucleotide shorter than its equivalent in the sequence listing and in Table II of the description. This renders claims 20-22 unclear.

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(71) Applicants *(for all designated States except US)*: STICHTING VOOR DE TECHNISCHE WETENSCHAPPEN [NL/NL]; P.O. Box 3021, NL–3502 GA Utrecht (NL). SEED CAPITAL INVESTMENTS–2 (SCI–2) B.V. [NL/NL]; Bernadottelaan 15, NL–3527 GA Utrecht (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: VAN EMBDEN, Johannes, Dirk, Anthonie [NL/NL]; Van Limburg Stirumstraat 15, NL–3581 VB Utrecht (NL). SCHOULS, Leendert, Marinus [NL/NL]; IJsselsteen 47, NL–3961 GB Wijk Bij Duurstede (NL). JANSEN, Rudolph [NL/NL]; Golfpark 149, NL–8241 AC Lelystad (NL).

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Published

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(54) Title: A METHOD OF INTERSTRAIN DIFFERENTIATION OF BACTERIA

(57) Abstract

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INTERNATIONAL SEARCH REPORT

Int[illegible]tional Application No
PCT/NL 98/00186

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 31569 A (NEDERLANDEN STAAT ;EMBDEN JOHANNES DIRK ANTHONIE (NL); SCHOULS LEE) 23 November 1995 cited in the application see the whole document ---	1,3-6, 10-14, 17-25
X	KAMERBEEK J ET AL.: "Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 35, no. 4, 1997, pages 907-914, XP002091620 cited in the application see the whole document ---	1,3-6, 10-14, 17-25

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 1999	16/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Knehr, M

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 98/00186

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	GROENEN P M ET AL.: "Nature of DNA polymorphism in the direct repeat cluster of Mycobacterium tuberculosis; Application for strain differentiation by a novel typing method" MOLECULAR MICROBIOLOGY, vol. 10, no. 5, 1993, pages 1057-1065, XP002091621 cited in the application see the whole document	1,3-6, 10-14, 17-25
X	SOOLINGEN VAN D ET AL: "USE OF VARIOUS GENETIC MARKERS IN DIFFERENTIATION OF MYCOBACTERIUM BOVIS STRAINS FROM ANIMALS AND HUMANS AND FOR STUDYING EPIDEMIOLOGY OF BOVINE TUBERCULOSIS" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 32, no. 10, October 1994, pages 2425-2433, XP000647581	17-19
Y	see the whole document	8-12,15, 16
X	US 5 691 136 A (KOEUTH THEARITH ET AL) 25 November 1997	1,8-12, 15-19
Y	see the whole document	20-22
Y	KLENK H-P ET AL.: "The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon Archaeoglobus fulgidus" NATURE, vol. 390, 1997, pages 364-370, XP002091622 cited in the application see abstract; table 1	20-22
Y	MOJICA F J M ET AL.: "Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferx volcanii and could be involved in replicon partitioning" MOLECULAR MICROBIOLOGY, vol. 17, no. 1, 1995, pages 85-93, XP002091623 cited in the application see abstract see page 85, column 2, paragraph 1 - page 87, column 1, paragraph 2; figure 1	1,3-6

-/--

2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 98/00186

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIEBL W ET AL.: "Analysis of a Thermotoga maritima DNA fragment encoding two similar thermostable cellulases, CelA and CelB, and characterization of the recombinant enzymes" MICROBIOLOGY, vol. 142, 1996, pages 2533-2542, XP002091624 see abstract see page 2536, column 1, paragraph 3 - page 2537, column 1, paragraph 1; figures 3,4 ---	1,3-6
Y	SHANGKUAN Y-H ET AL.: "Diversity of DNA sequences among Vibrio cholerae O1 and non-O1 isolates detected by whole-cell repetitive element sequence-based polymerase chain reaction" JOURNAL OF APPLIED MICROBIOLOGY, vol. 82, no. 3, 1997, pages 335-344, XP002091625 see the whole document ---	1,3-6, 8-12,15, 16
A	SOOLINGEN VAN D ET AL: "COMPARISON OF VARIOUS REPETITIVE DNA ELEMENTS AS GENETIC MARKERS FOR STRAIN DIFFERENTIATION AND EPIDEMIOLOGY OF MYCOBACTERIUM TUBERCULOSIS" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 31, no. 8, August 1993, pages 1987-1995, XP000647582 cited in the application ---	
A	VERSALOVIC J ET AL.: "Distribution of repetitive DNA sequences in eubacteria and application to fingerpriting of bacterial genomes" NUCLEIC ACIDS RESEARCH, vol. 19, no. 24, 1991, pages 6823-6831, XP002091626 see the whole document -----	

2

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/NL 98/ 00186

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2,7
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Subclaims 2 and 7 revealed to be not searchable since they are unclear and lack clarity (claim 2) and comprise no technical features suitable to perform any search (claim 7).

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/NL 98/00186

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9531569 A	23-11-1995	AU 690118 B	23-04-1998
		AU 6858294 A	05-12-1995
		EP 0760005 A	05-03-1997
		JP 10500011 T	06-01-1998
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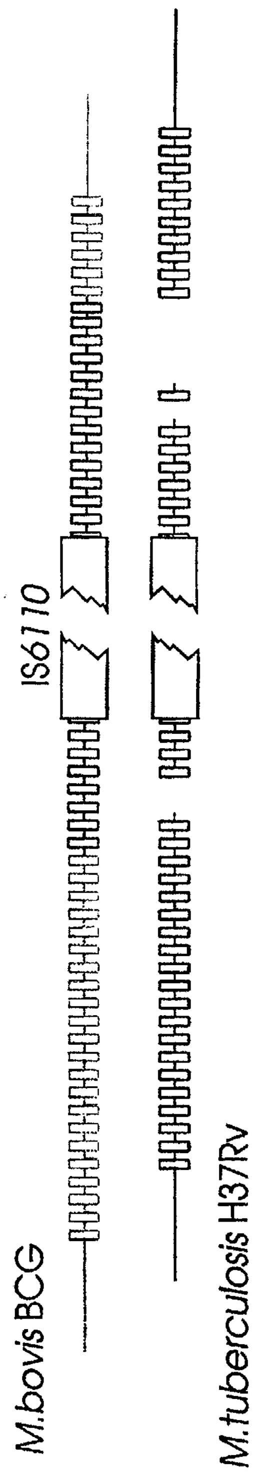
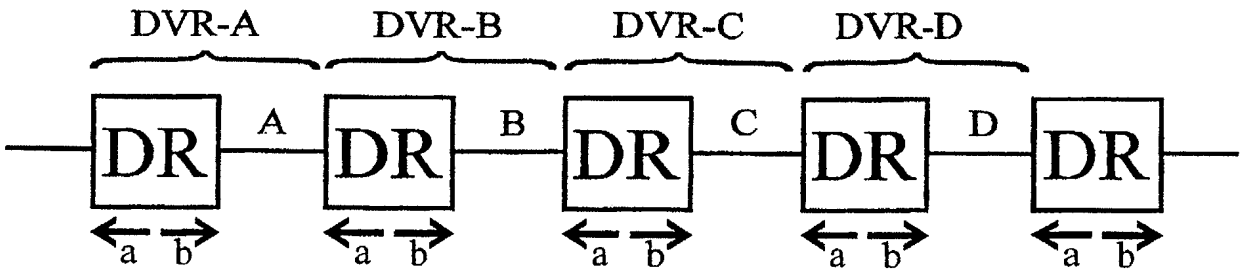
		CA 2121696 A	29-04-1993
		EP 0610396 A	17-08-1994
		WO 9308297 A	29-04-1993
		US 5523217 A	04-06-1996

Fig 1



Consensus DR sequence: GTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAAC

Fig 2



amplification products :

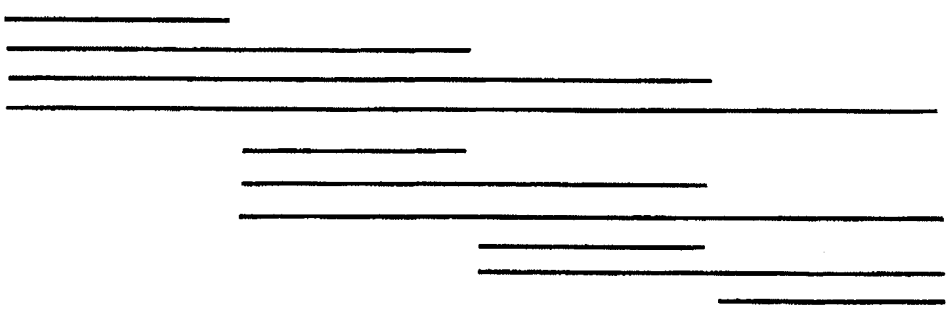
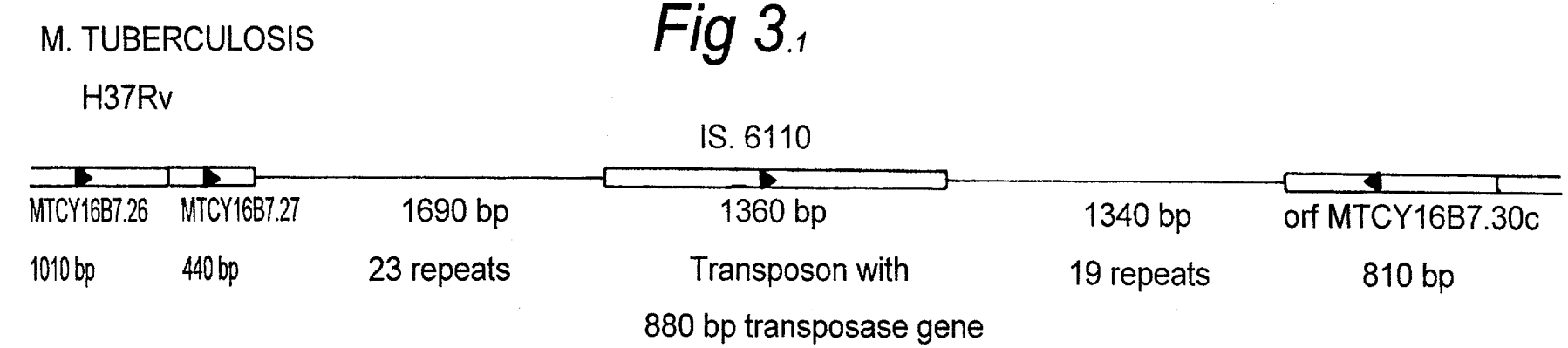


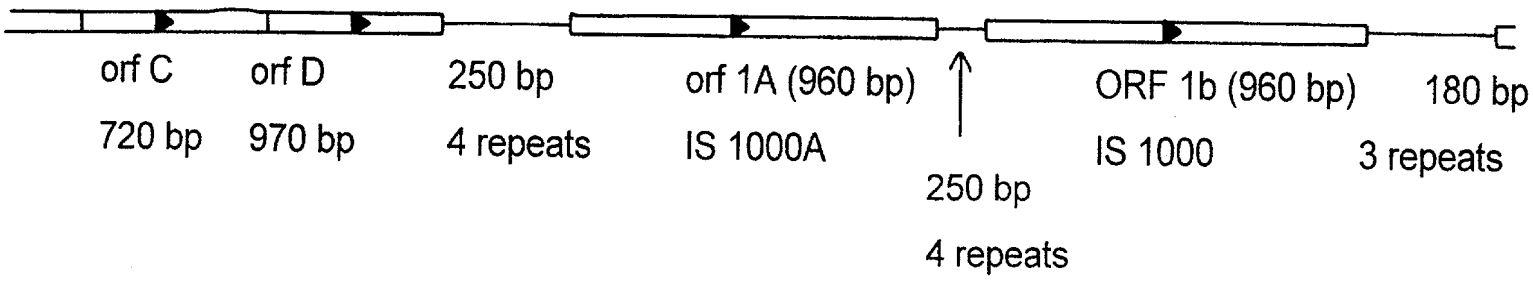
Fig 3.1



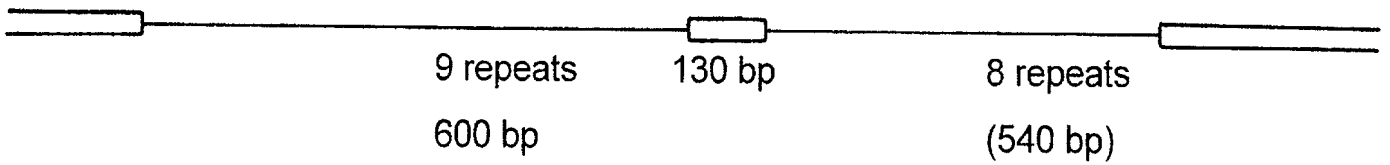
WO 99/51771
PCT/NL98/00186

THERMUS AQUATICUS THERMOPHILUS
HB8 ATCC 27634

Fig 3.2



ANABAENA sp. PCC 7120



HALOFERAX MEDITERRANEI

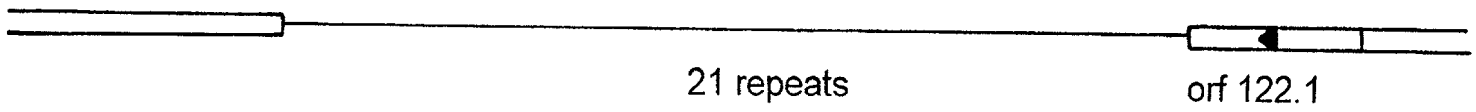
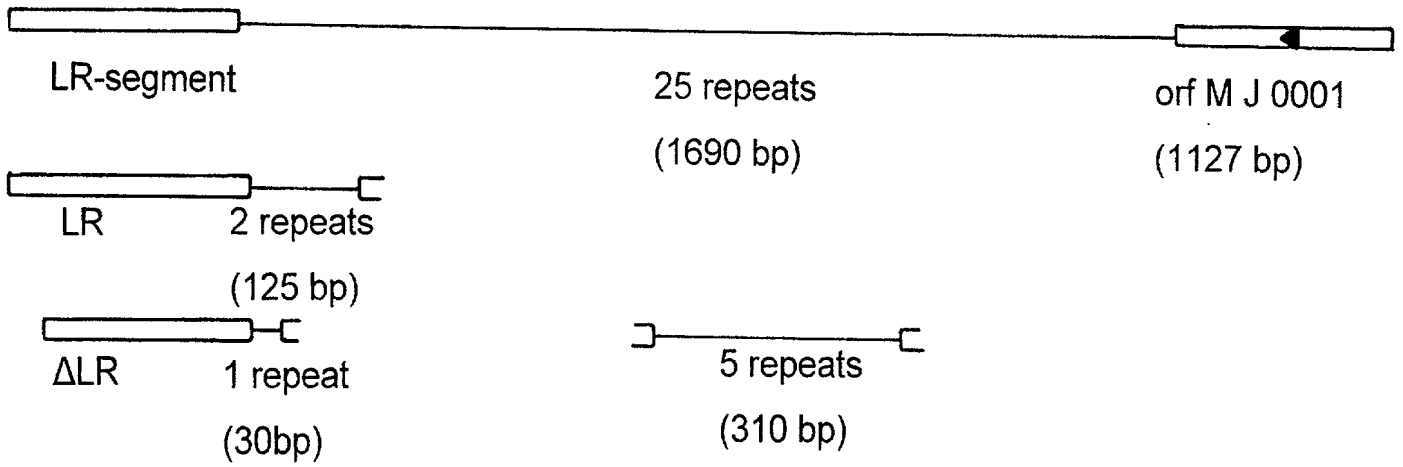


Fig 3.3

METHANOCOCCUS JANNASCHII



METHANOBACTERIUM THERMOAUTOTROPHICUM

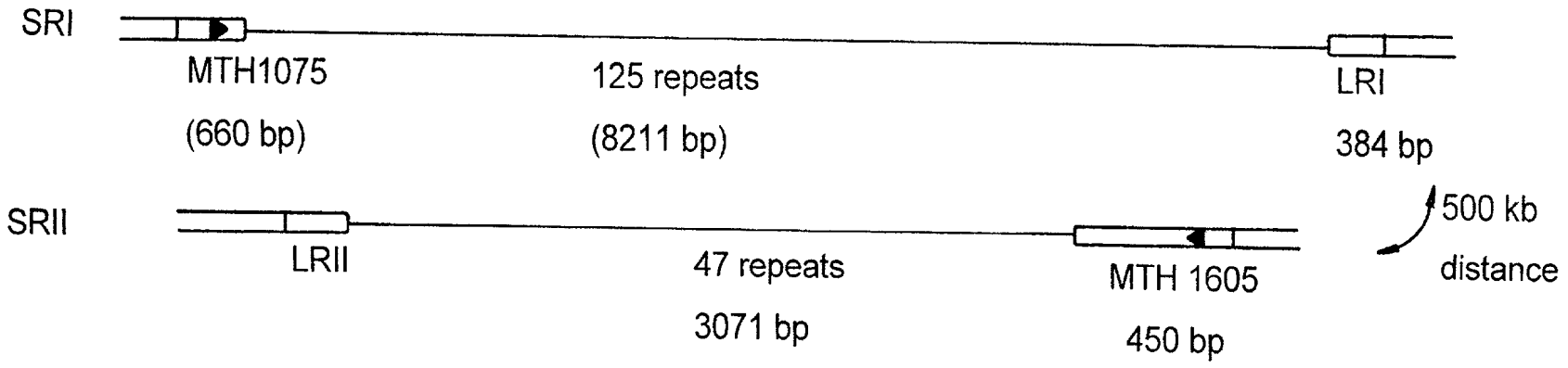


Fig 3.4

THERMOTOGA MARITIMA

9 repeats (570 bp)

cel A (873 bp)

cel B (824 bp)

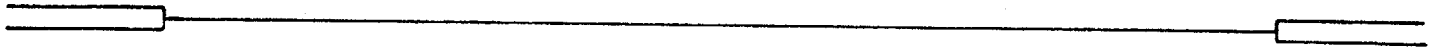
ARCHAEOGLOBUS FULGIDUS

SRI-A

60 repeats (4066 bp)

orf 3

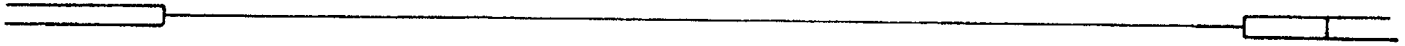
SRI-B



tRNA GAG

48 repeats (3222 bp)

SRII

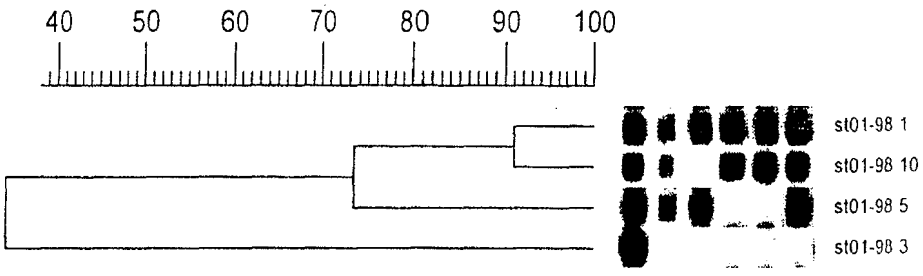


42 repeats 3174 bp

tRNA TGT

Fig 4

Fig 5



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(11) International Publication Number: **WO 99/51771**

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(74) Agent: DE BRUIJN, Leendert C.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL–2502 LS The Hague (NL).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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09/647596

532 Rec'd PCT/PTC 03 OCT 2000

WO 99/51771 PCT/NL98/00186

A method of interstrain differentiation of bacteria.

Summary of the invention

The subject invention lies in the field of interstrain differentiation of bacteria. A general method has been developed with which various types of bacteria can be differentiated into separate individual strains. Thus in particular in the clinical setting this method can suitably be used to determine what strain of bacterium is present in a sample. This new method is applicable for discerning between various strains of both Gram negative and Gram positive types of bacteria.

Background of the invention

Previously we had disclosed a method called oligotyping for interstrain differentiation of *Mycobacterium tuberculosis* strains in WO95/31569. It was stated in this document that one of the key factors in the control of tuberculosis is the rapid diagnosis of the disease and the identification of the sources of infection. *M. tuberculosis* strain typing has already proved to be extremely useful in outbreak investigations (6, 14, 31) and is being applied to a variety of epidemiologic questions in numerous laboratories. Traditionally, laboratory diagnosis is done by microscopy, culturing of the micro-organism, skin testing and X-ray imaging. Unfortunately, these methods are often not sensitive, not specific and are very time-consuming, due to the slow growth rate of *M. tuberculosis*. Therefore, new techniques like in vitro amplification of *M. tuberculosis* DNA have been developed to rapidly detect the micro-organism in clinical specimens (14). The ability to differentiate isolates of *M. tuberculosis* by DNA techniques has revolutionarized the potential to identify the sources of infection and to establish main routes of transmission and risk factors for acquiring tuberculosis by infection (1,3-10, 14, 16, 19-22, 25, 26, 27-33). The use of an effective universal typing system will allow strains from different geographic areas to be compared and the movement of individual strains to be tracked. Such data may provide important insights and identify strains with particular problems such as high infectivity, high virulence and/or multidrug resistance. Analysis of large numbers of isolates may provide answers to long-standing questions regarding the efficacy of BCG vaccination and the

frequency of reactivation versus reinfection.

The same problems identified for M. tuberculosis are inherent in differentiation of numerous other bacteria. The problems specifically arise for potentially epidemic pathogens and for bacteria that infect hospitals. A more rapid and simple typing method is required. Preferably the testing methods for various bacteria will occur in the same manner ensuring routine use for all types of bacteria for which testing is required. Preferably a test that can be carried out by non specialised personnel using little laboratory space and time is sought after.

The method disclosed in WO95/31569 is based on the DNA polymorphism found at a unique chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in *M. tuberculosis* complex bacteria. This locus was discovered by Hermans et al. (15) in *M. bovis* BCG, the strain used worldwide to vaccinate against tuberculosis. The DR region in *M. bovis* BCG consists of Directly repeated sequences of 36 base pairs, which are interspersed by non-repetitive DNA spacers, each 35 to 41 base pairs in length (15). The number of copies of the DR sequence in *M.bovis* BCG was determined to be 49. In other strains of the *M. tuberculosis* complex the number of DR elements was found to vary (15). The vast majority of the *M. tuberculosis* strains contain one or more IS6110 elements in the DR containing region of the genome.

It has been shown (12) that the genetic diversity in the DR region is generated by differences in the DR copy number, suggesting that homologeous recombination between DR sequences may be a major driving force for the DR-associated DNA polymorphism (12). The high degree of DNA polymorphism within a relatively small part of the chromosome makes this region well-suited for a PCR-based fingerprinting technique.

Figure 1 depicts the structure of the DR region of *M. bovis* BCG as determined previously by Hermans et al. and Groenen et al. (12, 15). For the sake of convenience we will designate a DR plus its 3'adjacent spacer sequence as a "Direct Variant Repeat" (DVR). Thus, the DR region is composed of a discrete number of DVR's, each consisting of a constant part (DR) and a variable part (the spacer).

The method disclosed in WO95/31569 is based on a unique method of in vitro amplification of DNA sequences within the DR region and the hybridisation of the amplified DNA with multiple, short synthetic oligomeric DNA sequences based on the sequences of the unique spacer DNA's within the DR region (figure 2). This differs from previous PCR methods in the use of a set of primers with both primers having multiple

priming sites as opposed to having one of the primers bind to a fixed priming site such as to a part of IS6110. Because *M. tuberculosis* complex strains differ in the presence of these spacer sequences, strains can be differentiated by the different hybridisation patterns with a set of various spacer DNA sequences.

The method consists of in vitro amplification of nucleic acid using amplification primers in a manner known per se in amplification reactions such as PCR, LCR or NASBA, wherein a pair of primers is used comprising oligonucleotide sequences sufficiently complementary to a part of the Direct Repeat sequence of a microorganism belonging to the *M. tuberculosis* complex of microorganisms for hybridisation to a Direct Repeat to occur and subsequently elongation of the hybridized primer to take place, said primer being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction. Due to the multiple presence of Direct Repeats in the microorganisms to be detected the use of such primers implies that all the spacer regions will be amplified in an efficient manner. In particular it is not necessary for extremely long sequences to be produced in order to obtain amplification of spacers located at a distance from the primer. With the instant selection of the primer pairs a heterogenous product is obtained comprising fragments all comprising spacer region nucleic acid. Subsequently the detection of the amplified product can occur simply by using an oligonucleotide probe directed at one or more of the spacer regions one wishes to detect. In order to avoid hindrance in the amplification reactions the primers can have oligonucleotide sequences complementary to non-overlapping parts of the Direct Repeat sequence so that when both primers hybridize to the same Direct Repeat and undergo elongation they will not be hindered by each other. In particular to avoid any hindrance during elongation reactions when one primer DRa is capable of elongation in the 5' Direction and the other primer DRb is capable of elongation in the 3' Direction the DRa is selected such that it is complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary. The primer used must have an oligonucleotide sequence capable of annealing to the consensus sequence of the Direct Repeat in a manner sufficient for amplification to occur under the circumstances of the particular amplification reaction. A person skilled in the art of amplification reactions will have no difficulty in determining which length and which degree of homology is required for good amplification

reactions to occur. The consensus sequence of the Direct Repeat of microorganisms belonging to the *M. tuberculosis* complex is given in sequence id. no. 2 and in figure 1.

In addition to what has already been disclosed in WO95/31569 we also determined the spoligotypes of M. tuberculosis strains which were subcultured for many months both in the laboratory and in guinea pigs. The strains selected for this purpose were those used in a previous study on the stability of IS 6110 (2). All subcultured strains displayed the identical spoligotype patterns compared with the primary cultures thus indicating the pace of the molecular clock in this instance is slow enough for use in epidemiology of the disease.

Because of the large success and simplicity of the method for Mycobacterium tuberculosis strain differentiation and in view of problems in strain differentiation with other microorganisms we used the Direct Repeat consensus sequence to screen data bases with nucleic acid sequences from other microorganisms. Unfortunately no further matches were found. The Direct Repeat sequence appeared to be unique for the Mycobacterium tuberculosis as did their spacer sequences. As to date no function had actually been attributed to the Direct Repeat sequence it was unexpected that the sequence was universally distributed amoung other types of microorganisms. Such would at best be expected if the sequence had a function that was required also in other organisms.

Description of the invention

Notwithstanding the negative result after screening with the Direct Repeat consensus sequence we considered further analysis of known sequences by looking for a pattern in the nucleic acid sequences of other microorganisms reminiscent of the Direct Repeat-spacer pattern in Mycobacterium tuberculosis. Quite unexpectedly we found using a specifically designed computer programme that such patterns existed in a large number of other microorganisms with a broad range of genera. It appears that the DR-like sequences are very common in prokaryotes. They are however noticeably absent in eukaryotes. Chapter III of Bergeys Determinative Manual of Bacteriology Ninth edition (11) provides a table of characteristics for distinguishing prokaryotes from eukaryotes i.e. distinguish bacterium from microoscopic eukaryotes in the shape of mold, yeast, algae or protozoans.

All bacterial sequences analysed revealed the presence of such

a sequence structure and thus the oligotyping method illustrated for Mycobacterium tuberculosis can be applied for differentiating between all strains of bacteria. It was totally unexpected that a consensus structure of this type could be universally found. The Direct Repeat sequences themselves are different between different genera but the general framework of a cluster of Direct Repeat sequences, separated by a number of non repetitive spacers is universally present in bacterial genomes. Considering the fact that thusfar no function has been attributed to such a region in Mycobacterium tuberculosis or in fact for any of the sequences comprising Direct Repeat like regions in any other bacteria for which such sequences had been described this is remarkable.

Bacteria can be divided into Archaebacteria and Eubacteria. The eubacteria in turn can de distinguished into Gram-negative and Gram-positive bacteria with cell walls and Eubacteria lacking cell walls. Chapter IV of Bergeys determinative Manual of Bacteriology Ninth edition (11) reveals the characteristics for each group. Over a wide range of the subgroups in these 4 groups we have found the presence of the consensus structure i.e. the presence of DR-like loci. The IV groups have been subdivided by Bergey into more than 30 subgroups. We have examples in Groups 3,4,5 and 6, Group 11, 17, 31, 32, 33. The method according to the invention is particularly of interest for the bacteria that are pathogenic for humans. Group 4 comprises Gram negative bacteria. Genera from Group 4 are Legionella (which can cause pneumonia) and Legionnaires disease, the genus Neisseria (of which Neisseria meningitidis is well known as causative agent of meningitis and of which Neisseria gonorrhoeae is another example), the genus Pseudomonas (renown for hospital infections) and the genus Bordetella (of which Bordetella pertussis is well known as causative agent of whooping cough). In Group 5 bacteria as defined in Bergeys Manual the Enterobacteriacae form a family of 30 genera. These bacteria form a particularly interesting group of Gram negative bacteria that infect humans. Suitable examples of genera from this family are Enterobacter, Escherichia, Shigella, Salmonella, Serratia, Klebsiella and Yersinia. Other less well known pathogenic Enterobacteriacae genera are Cedeca, Citrobacter, Kluyvera, Leclercia, Pantoea, Proteus, Providencia and Hafnia. Other Group 5 families are Pasteurellaceae with the genus Haemophilus and the family Vibrionaceae with the genus Vibrio. Haemophilus influenzae is a leading cause of meningitis in children and also other septicemia conditions. Vibrio cholerae is the causative agent of cholera, V. parahaemolyticus can cause

food poisoning and V. vulnificus causes highly fatal septicemia.

Of the Enterobacteriacae Shigella, Escherichia and Salmonella are best known and difficult to differentiate. Shigella is an intestinal pathogen of humans causing bacillary dysentery. Well known strains are S. dysenteriae, S. flexneri, S. boydii, S. sonnei. The genus Salmonella is well known for food poisoning. Well known Salmonella strains are S. typhimurium, S. arizona, S. choleraesuis, S. bongori. Salmonella are also causative agents of typhoid fever, enteric fevers, gastroenteritis and septicemia. The genus Serratia bacteria are opportunistic pathogens for hospitalized humans causing septicemia and urinary tract infections. Examples are S. liquefaciens and S. marcescens. Of the Escherichia E. coli is best known as major cause of urinary tract infections and nosocomial infections including septicemia and meningitis. Other species are usually associated with wound infections.

Enterobacter constitutes a problem genus of opportunistic pathogens causing burn wound and urinary tract infections occasionally also meningitis and septicemia. Well known species are E. cloacae, E. sakazakii, E. aerogenes, E. agglomerans, E. gergoviae. Klebsiella are also causative agents of bacteriemia, pneumonia, urinary tact and other human infections in urological, neonatal, intensive care and geriatric patients. Klebsiella pneumoniae and K. oxytoca are examples of species in the genus.

Particularly interesting from a clinical point of view are also the Gram positive pathogenic bacteria. The genera Streptococcus and Staphylococcus form examples of such bacteria. Streptococcus pneumoniae, Streptococcus pyogenes and Staphylococcus aureus are examples thereof. Of the mentioned groups and genera the pathogenic bacteria are of interest. These bacteria are dangerous when infecting hospitals in particular.

Due to the increasing incidence of infection differentiation of potentially epidemiological organisms is also of interest. Such organisms comprise Bordetella pertussis and Neisseria menigitidis the causative organism of meningitis is of particular interest. Quite specifically pathogenic bacteria infecting hospitals and bacteria capable of causing epidemics are targets for the differentiation method according to the invention.

The invention consists of a method of in vitro amplification of nucleic acid using amplification primers in a manner known per se, in amplification reactions such as PCR, LCR or NASBA, wherein a pair of primers is used comprising oligonucleotide sequences sufficiently

complementary to a part of the Direct Repeat sequence of a bacterium other than a microorganism belonging to the M tuberculosis complex of microorganisms for hybridisation to a Direct Repeat to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction, wherein the Direct Repeat is a sequence with a length between 20-50 base pairs which occurs 5-60 times in a contiguous region of the bacterial genome, whereby the Direct Repeat sequences are separated by spacer sequences with a length of between 20-50 nucleotides, said spacer sequences being non repetitive. By using the programme Patscan e.g. on the nucleic acid data bases for microorganism genomic sequences such motifs and thus also the identities of the various species specific Direct Repeats and the corresponding spacer sequences can be obtained. In the Patscan programme the Direct Repeat can be designated p1 with a length between 20-50 basepairs then search for p1 20-50 basepairs downstream of p1. Thus this pattern in Patscan is described as p1=(20..50)(20..50)p1(20..50)p1. The length of the sequences can be varied as can the intermediate distance and the number of times the Direct Repeat has to occur. A Direct Repeat can often have a length of 30-40 base pairs with a spacer length of 35-45 base pairs. Basically we looked for a stretch of identical repeat sequences interspersed by spacer sequences which do not necessarily share much of their sequence with the Direct Repeat of *M. tuberculosis*. The patscan programme is freely accessible at the Internet site:http://www-c.mcs.anl.gov/home/overbeek/-PatScan/HTML/patscan.html. The programme was written by Ross Overbeek Mathmatics and Computer Science Division Argonne National Laboratory Building 221 Room D-236 9700 S. Cass Avenue Arginne IL 60439 USA.

Most of the Repeats exhibit one or more of the following characteristics. they end with a sequence similar to GAAAC i.e. exhibit at least 3 of the nucleotides of this consensus sequence at the terminus. preferably 4 or 5, start with CTTTG, have stretches of 3-4 identical bases. The termini can for example be selected from GAAAC, GAAXXC GAACTC, GXAAC, GCAAC, GAAA, GAAXC, GAAGC and AAAC. Suitable Termini are provided in Table II.

Organisms as diverse as the Archaebacteria e.g. Methanococcus jannasschi (Group 31), Haloferax mediterranei (Group 33), the cyanobacteria Calotrix (Group 11), and Anabeana (Group 11), and purple bacteria e.g. E.coli (Group 5), Mycobacterium tuberculosis (Group 21) and

Thermus thermophilus (Group 4), Archaeoglobus (Group 32) and Thermotoga (Group 6) were found to possess DR-like sequences upon analysis of their genomes using the Patscan programme. In the subsequent study of literature from which these data were derived it also became clear from Southern blots that the Repeat sequences were also found in related species.

With regard to the genetic organisation the structures of the DR-like loci in the microorganisms is rather variable (figure 3). In M. tuberculosis the DR locus is large and in most isolates it is disrupted by an insertion element. This is also the case in T. thermophilus, however here the number of DVR's is only 11 and the DR locus is disrupted by two insertion elements. In E. coli K12 2 DR loci are present separated by approximately 22kb; in Anabaena the locus is of intermediate size and interrupted by a 130 bp sequence of unknown function or origin. In H. mediterranei the DR locus is of intermediate size and not disrupted, however there is evidence for a second DR locus on one of the mega plasmids found in this organism. In M. jannaschii there is one locus of intermediate size but at several other positions in the genome one or a few other DVR's are found. In most cases the DVR's are linked to a so-called Long Repeat (LR) element of unknown function. Also in M. jannaschii mega plasmids are found but in contrast to H. mediterranei they do not contain DR sequences.

Accession numbers for the sequences of various organisms for which the DR like loci have been found are provided here. For E. coli and Shigella M27059, M27060, U29579, U29580 and M18270. The relevant portions of the sequences are also disclosed by Blattner for E. coli. Nakata et al reveal in the Journal of Bacteriology (13) that downstream of the iap region a sequence of 29 bases appears 14 times 32 or 33 base pairs apart. Nucleotide sequences hybridizing to the 29 base pair sequence were also detected in Shigella dysenteriae and Salmonella typhimurium.

A DR-like sequence was found in the contig 214 of S. pyogenes M1(ATCC 700294) of the genome sequencing project of the University of Oklahoma. Further research into this DR-like sequence in other S. pyogenes revealed spacer polymorphism. The DR regions of eight S. pyogenes isolates were studied. The DR regions were isolated by PCR using primers that were derived from the database (University of Oklahoma, serotype M1 ATCC 700294. The sequence data is available under http://www.genome.ou.edu. This strain contains seven repeats and six spacers.

Five of the isolates gave a PCR product, these were a M2 strain, a M4 strain and three M1 strains. The M4 strain contained only a single repeat sequence that was flanked by the same sequences as the ATCC 700294. The M2 strain sequencing did not work, but the size of the PCR fragment indicated that two repeats are present. The three M1 strains were all the same, they contained four repeats and three spacers. The repeats were identical to ATCC 700294, while one of the spacers was identical to ATCC 700294 and two were different.

These studies on *S. pyogenes* show that the DR regions have conserved spacers and repeat sequences.

The Salmonella genomic sequence as sequenced by the University of Washington St Louis has also revealed the presence of DR-like sequences. The DR exhibits high homology with the Direct Repeat of E. coli. One of the contigs revealed 7 Repeats and 6 spacers.

A panel of five *E. coli* isolates and three *Shigella* strains were studied. The five *E. coli* isolates were selected to have an optimal diversity, they were isolated from different species or geographic regions. The *Shigella* strains are considered separate (sub)species. See Table 1. The isolates were obtained from the collection of Dr. Wim Gaastra.

Table 1

species		description	DRI*	DRII*
E. coli	184	American isolate	Southern	PCR
	358	human urinary tract	Southern	Southern
	968	mastitis	Southern	PCR
	1008	chicken	PCR	PCR
	1732	human intestine	Southern	PCR
Shigella disenteriae		593	Southern	PCR
	sonnei	595	Southern	PCr
	boydil	603	Southern	PCR

* The DR regions were identified by Southern blot of genomic DNA and DRI and DRII regions of *E. coli* K12. When PCR is indicated the DR regions were identified by the Southern and the PCR. This PCR was done with primers derived from the K12 sequence.

The DRI and DRII sequences that could be amplified by PCR were

cloned and sequenced. Somehow the DRI regions could not be amplified by PCR using the primers designed on the K12 sequence, while the Southern data demonstrate that DRI is present. Apparently, the recognitions sites for the primers are polymorphic. The sizes of the DRII regions were found to vary greatly between these isolates. The smallest was a single repeat in the *S. sonnei* strain and the largest was a repeat cluster of at least 15 repeats in *E. coli* isolate 1008. The sequences of the repeats were highly conserved between these isolates. The *S. typhimurium* data is obtainable from the Internet http://genome.wustl.edu/gsc/-bacterial/salmonella.html.

The spacer sequences almost all were unique. Approximately 40 spacers have been sequenced and only three of them were already known from a previously sequenced DR region. This indicates a high number of different spacer sequences in *E. coli*.

Accession number X73453 provides the Halerofax mediterranei sequence. The sequence can also be found in Molecular Microbiology 17 of 1995 in an article by Mojica et al. (17). The Repeat sequence has also been found in related species.

The genomic project of the Methanococcus jannaschii reveals a DR-like sequence as is apparent from the Bult et al article in Science 273 of 1996 (18). The Accession number is U67459 i.a.

Accession number X87270 for Anabeana sp reveals 17 spacers and a LTRR element. These elements also occur in related species of cyanobacteria such as Calotrix. The sequence data are provided by Masepohl et al in BBA 1307 1996 (23).

Accession number AE000782 for Archaeoglobus fulgidus reveals three DR-like Repeats with the same Repeat sequence and the this has a slightly larger but closely related Repeat. The Repeats are present 20-30 times. The spacers are unique sequences. H.P. Klenk discloses sequence data in Nature 390 1997 (24).

The invention also covers a method of detection of a bacterium, said bacterium not belonging to the M. tuberculosis complex of microorganisms said method comprising

1) amplifying nucleic acid from a sample with the amplification method according to any of the preceding described embodiments of the amplification method according to the invention, followed by
2) carrying out a hybridisation test in a manner known per se, wherein the amplification product is hybridised to an oligonucleotide probe or a plurality of different oligonucleotide probes, each

oligonucleotide being sufficiently homologous to a part of a spacer of the Direct Region of the bacterium to be determined for hybridisation to occur to amplified product if such spacer nucleic acid was present in the sample prior to amplification, said hybridisation step optionally being carried out without prior electrophoresis or separation of the amplified product.

3) detecting any hybridised products in a manner known per se.

The method can be carried out in a manner such that the hybridisation test is carried out using a number of oligonucleotide probes, said number comprising at least a number of oligonucleotides probes specific for the total spectrum of bacteria it is desired to detect. In a suitable embodiment of a method according to the invention the oligonucleotide probe is at least seven oligonucleotides long and is a sequence complementary to a sequence selected from any of the spacer sequences of the Direct Repeat region of the bacterium to be determined or is a sequence complementary to fragments or derivatives of said spacer sequences, said oligonucleotide probe being capable of hybridising to such a spacer sequence and comprising at least seven consecutive nucleotides homologous to such a spacer sequence and/or exhibiting at least 60% homology, preferably exhibiting at least 80% homology with such a spacer sequence.

Preferably the method according to the invention is carried out to determine the presence and nature of a pathogenic bacterium selected from the group of Gram negative bacteria of Groups 4 and 5 of Bergeys Determinative Manual of Bacteriology ninth edition. Of particular interest due to damage caused by such pathogens are bacteria belonging to the families Enterobacteriaceae, Pasteurellaceae and Vibrionaceae of Group 5, most specifically the Enterobacteriaceae. Also of interest are the Gram positive bacteria of Group 17. Suitable examples of genera of the pathogenic bacterium to be detected from the group of Gram negative bacteria of Bergeys Determinative Manual of Bacteriology ninth edition are Eschericchia, Shigella, Salmonella, Klebsiella, Enterobacter, Yersinia, Serratia, Haemophilus, Vibrio, Legionella, Neisseria, Pseudomonas and Bordetella. For the group of Gram positive bacterial genera Staphylococcus and Streptococcus are targets for the differentiation method.

Suitably in a method according to the invention for differentiating the type of bacterium in a sample, said bacterium not belonging to the M. tuberculosis complex the hybridisation pattern is

compared with that obtained with a reference. Such a reference can be the hybridisation pattern obtained with one or more known strains of the bacterium to be determined in analogous manner as the strain to be determined. Alternatively the reference is a source providing a list of spacer sequences and sources thereof, such as a data bank. Table II exhibits some suitable examples of sequences that occur as Direct Repeat sequences according to the invention for the genera illustrated.

Table II

Species	Repeat sequence	Reference	EMBL/Genbank accession number
Mycobacterium tuberculosis	GTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAAC	Hermans et al.	
Escherichia coli	CGGTTTATCCCCGCTGGCGCGGGGAACTC	Nakata et al.J.Bact.171 3553-3556 (1989)	M27059 and M27060
Shigella disenteriae	CGGTTTATCCCCGCTGGCGCGGGGAACTC	our own data	
Shigella sonnei	CGGTTTATCCCCGCTGGCGCGGGGAACTC	our own data	
Shigella boydii	CGGTTTATCCCCGCTGGCGCGGGGAACTC	our own data	
Salmonella enteritidis	CGGTTTATCCCCGCTGGCGCGGGGAACTC	our own data	
Serratia marcescens	CGGTTTATCCCCGCTGGCGCGGGGAACTC	our own data	
Salmonella typhimurium	CGGTTTATCCCCGCTGGCGCGGATACAC	contig 70A06 of the typhimurium genome project.Univ. of Washington St. Louis	
Streptococcus pyogenes	GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACT	Contig 214 of the S. pyogenes genome project University of Oklahoma	

(Table II)

Species	Repeat sequence	Reference	EMBL/Genbank accession number
Thermus aquaticus thermophilus	AATCCCCTTACGGGGCTCAATCCCTTGCAA	Ashby et al. Plasmid 24 1-11 (1990)	M33159
Thermotoga maritima	GTTTCAATACTTCCTTAGAGGTATGGAAAC	Liebl.et al. Microbiol.142 2533-2542 (1996)	Z69341
Anabaena	GTTTTAACTAACAAAAATCCCTATCAGGGATTGAAAC	Masepohl BBA 1307 26-30 1996	X87270
Calotrix	GTTTAAACTTTATAAAATCCCTTTTAGGGATTGAAAC	idem	Z47161
Haloferax mediterranei	GTTACAGACGAACCCTAGTTGGGTTGAAGC	Mojica et al.Molec.Microbiol.17 85-93 (1995)	X7453
Methanococcus jannaschii	AATTAAAATCAGACCGTTTCGGAATGGAAA	Bult et al.Science 273 1058-1073 (1996)	U67459 (for the large DR-like region)
Methanobacterium	ATTTCAATCCCATTTTGGTCTGATTTTAAC	Smith et al. J. Bact. 179 7135-7155 (1997)	AE000920 (for R2)
thermoautotrophicum	GTTAAAATCAGACCAAAATGGGATTGAAAT		AE000878 (for R1)
Archaeoglobus fulgidus	CTTTCAATCCCATTTTGGTCTGATTTCAAC	Klenk et al. Nature 390 364-370 (1997)	AE000782 (whole genome)

Not only the above methods fall within the scope of the invention but also specifically selected primer pairs for carrying out such a method. A pair of primers according to the invention is a pair wherein both primers comprise oligonucleotide sequences of at least 7 oligonucleotides and are sufficiently complementary to a part of the Direct Repeat sequence of the microorganism E. coli for hybridisation to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction and wherein sufficiently complementary means said oligonucleotide sequence comprises at least seven consecutive nucleotides homologous to such a Direct Repeat sequence and/or exhibits at least 60% homology, preferably at least 80% homology, most preferably more than 90% homology with the corresponding part of the Direct Repeat sequence. Suitable Direct Repeat sequences are provided in Table II. In particular such a primer pair can comprise one primer DRa capable of elongation in the 5' Direction and the other primer DRb capable of elongation in the 3' Direction with DRa being complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary, the Direct Repeat being present in the Direct Region of E. coli. Another suitable pair comprises primers with oligonucleotide sequences of at least 7 oligonucleotides and are sufficiently complementary to a part of the Direct Repeat sequence of the microorganism S. typhimurium for hybridisation to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction and wherein sufficiently complementary means said oligonucleotide sequence comprises at least seven consecutive nucleotides homologous to such a Direct Repeat sequence in particular the Sequence provided in Table II and/or exhibits at least 60% homology, preferably at least 80% homology, most preferably more than 90% homology with the corresponding part of the Direct Repeat sequence. In particular such a pair comprises one primer DRa capable of elongation in the 5' Direction and the other primer DRb capable of elongation in the 3' Direction with DRa being complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary, the Direct Repeat being present in the Direct Region of S. typhimurium.

Kits for carrying out a differentiation method according to any

of the described embodiments also fall within the scope of the invention. Such kits comprise a primer pair according to any of the described embodiments and optionally an oligonucleotide probe or a carrier, said carrier comprising at least 1 oligonucleotide probe specific for a spacer region of a bacterium to be determined said bacterium not belonging to M tuberculosis complex, preferably the oligonucleotide probe as defined, being an oligonucleotide probe of at least 10 nucleotides, preferably more than 12 nucleotides, in particular comprising between 12 to 40 nucleotides, said probe being sufficiently homologous to any of the spacer sequences or to fragments or derivatives of such spacer sequences to hybridise to such a spacer sequence, said oligonucleotide probe comprising at least 10 consecutive nucleotides homologous to such a spacer sequence and/or exhibiting at least 60% homology, preferably exhibiting at least 80% homology, most preferably exhibiting more than 90% homology with the corresponding part of the spacer sequence. Suitably a kit according to the invention comprises a data carrier with required reference patterns of the bacterial strain to be determined.

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DESCRIPTION OF THE FIGURES

Figure 1 depicts the structure of the DR region of *M. bovis* BCG as determined previously by Hermans et al. and Groenen et al. (12, 15). For the sake of convenience we will designate a DR plus its 3'adjacent spacer sequence as a "Direct Variant Repeat" (DVR). Thus, the DR region is composed of a discrete number of DVR's, each consisting of a constant part (DR) and a variable part (the spacer).

Figure 2 depicts multiple, short synthetic oligomeric DNA sequences based on the sequences of the unique spacer DNA's within the DR region.

Figure 3 shows the genetic organisation of the structures of the DR-like loci in various bacterial species.

► depicts the transcription direction of open reading frame (ORF)

For M. tuberculosis: MTCY 16B7.26, 27 and 30C are unknown genes/proteins.

For E. coli: iap gene function is alkaline phosphatase isozyme conversion. ORF f94, f305, YGCE and f223 are unknown genes/proteins.

For S. pyogenes: ORF1 and 2 are unknown genes/proteins.

For T. thermophilus: ORFC and D are unknown genes/proteins and ORF 1A and 1B are possibly transposases of IS elements 1000 and 1000A.

For Anabaena: No ORFs were annotated in the flanking sequences. The 130 bp insert is of unknown origin.

For Haloferax mediterranei: ORF21 is an unknown gene/protein. Probably another repeat cluster is also present on the megaplasmid pHM500.

For Methanococcus jannaschii: Comprises about 10 repeat clusters, the largest one of which comprises 25 repeats. All repeat clusters are coupled to a Long Repeat (LR) segment of 425bp. There are 18 LR's, some of which contain only one repeat. Smaller LR segments are also present, ΔLR. In one case, a cluster contains 5 repeats without LR (see ref. 18)

For M. thermoautrophicum: Two repeat clusters SRI and SRII flanked by

LRI. LRII. LRI and LRII are almost identical and are homologues of the LR segment of M. jannaschii. SRI and SRII are separated by 500 kb in the genome.

For Thermatoga maritima:CelA gene encodes cellulase: endo-1,4-beta-glucanase (EC 3.2.1.4) and CelB is also a cellulase exhibiting 58% identity with celA.

For Archaeoglobus fulgidus: The SRIA and SRIB repeat clusters have the same Repeat Sequence and the SRII Repeat Sequence is also clearly homologous. The SR clusters are separated by about 400bp. SRIB and SRII are located near tRNA genes. SRIA lies adjacent to an unknown ORF3.

Figure 4

Hybridization Patterns of 17 E. coli isolates. Thirty four different spacer oligonucleotides were covalently linked to a membrane and PCR amplified DNA of E. coli was hybridized as described (Kamerbeek et al. 1997), except that the primers used to amplify the DR locus were specific for the DR sequence from E. coli. Note the polymorphism observed in E. coli due to the strain-dependent presence or absence of spacer DNA.

Figure 5

Hybridization Patterns of 4 Salmonella typhimurium isolates. Six different spacer oligonucleotides were covalently linked to a membrane and PCR amplified Salmonella DNA was hybridized as described (Kamerbeek et al 1997), except that the primers used to amplify the DR locus were specific for the DR locus of E. coli. Note the polymorphism observed in Salmonella due to the strain-dependent presence or absence of spacer DNA.

CLAIMS

1. A method of in vitro amplification of nucleic acid using amplification primers in a manner known per se, in amplification reactions such as PCR, LCR or NASBA, wherein a pair of primers is used comprising oligonucleotide sequences sufficiently complementary to a part of the Direct Repeat sequence of a bacterium other than a microorganism belonging to the M tuberculosis complex of microorganisms for hybridisation to a Direct Repeat to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction, wherein the Direct Repeat is a sequence with a length between 20-50 base pairs which occurs 5-60 times in a region of the bacterial genome, whereby the Direct Repeat sequences are separated by spacer sequences with a length of between 20-50 nucleotides, said spacer sequences being non repetitive.

2. A method according to claim 1 wherein the Direct Repeat sequence is obtainable from screening a genomic bacterial nucleic acid sequence using the programme Patscan wherein the Direct Repeat is designated p1 with a length between 20-50 basepairs then p1 is sought 20-50 basepairs downstream of p1 as the pattern p1=(20..50)(20..50)p1(20..50)p1 or a variant thereof wherein the ranges of the nucleotide lengths are shorter and wherein the frequency of occurrence of the Direct Repeat can vary between 5 and 60.

3. A method according to claim 1 or 2 wherein the Direct Repeat has a length between 30-40 base pairs and the spacer has a length of 35-45 base pairs.

4. A method according to any of the preceding claims wherein the Direct Repeat has a terminus exhibiting at least 3 out of 5 nucleotides identical with the sequence GAAAC, preferably 4, said termini for example being selected from GAAAC, GAAXXC GAACTC, GXAAC, GCAAC, GAAA, GAAXC, GAAGC, AAAC.

5. A method according to any of the preceding claims wherein the Direct Repeat terminates with GAACTC, ATACAC, AAAACT, TTGCAA, GGAAAC,

TGAAAC, TGAAGC, TGGAAA, TTTAAC, TGAAAT or TTCAAC.

6. A method according to any of the preceding claims wherein the Direct Repeat has stretches of 3-4 identical bases.

7. A method according to any of the preceding claims wherein the Direct Repeat has a sequence such that it is not prone to loop formation or any other obvious secondary structure.

8. A method according to any of the preceding claims wherein the bacterium is a pathogenic bacterium selected from the group of Gram negative bacteria of Groups 4 and 5 of Bergeys Determinative Manual of Bacteriology ninth edition, in particular the families Enterobacteriaceae, Pasteurellaceae and Vibrionaceae of Group 5, most specifically the Enterobacteriaceae and the Gram positive bacteria of Group 17.

9. A method according to any of the preceding claims wherein the bacterium is a pathogenic bacterium selected from the group of Gram negative bacteria of Bergeys Determinative Manual of Bacteriology ninth edition of the genera Escherichia, Shigella, Salmonella, Klebsiella, Enterobacter, Yersinia, Serratia, Haemophilus, Vibrio, Legionella, Neisseria, Pseudomonas, Bordetella, Staphylococcus, Streptococcus and Acinetobacter.

10. A method according to any of the preceding claims, wherein said primers have oligonucleotide sequences complementary to non overlapping parts of the Direct Repeat sequence and such that the elongation reactions from each primer can occur without hindrance of the other when both primers hybridise to the same Direct Repeat and undergo elongation.

11. A method according to any of the preceding claims, wherein one primer DRa is capable of elongation in the 5' Direction and the other primer DRb is capable of elongation in the 3' Direction and DRa is complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary.

12. A method of detection of a bacterium, said bacterium not belonging to the M tuberculosis complex of microorganisms said method

comprising

1) amplifying nucleic acid from a sample with the method according to any of the preceding claims, followed by
2) carrying out a hybridisation test in a manner known per se, wherein the amplification product is hybridised to an oligonucleotide probe or a plurality of different oligonucleotide probes, each oligonucleotide being sufficiently homologous to a part of a spacer of the Direct Region of the bacterium to be determined for hybridisation to occur to amplified product if such spacer nucleic acid was present in the sample prior to amplification, said hybridisation step optionally being carried out without prior electrophoresis or separation of the amplified product.
3) detecting any hybridised products in a manner known per se.

13. A method according to claim 12, wherein the hybridisation test is carried out using a number of oligonucleotide probes, said number comprising at least a number of oligonucleotides probes specific for the total spectrum of bacteria it is desired to detect.

14. A method according to claim 12 or 13, wherein the oligonucleotide probe is at least ten oligonucleotides long and is a sequence complementary to a sequence selected from any of the spacer sequences of the Direct Repeat region of the bacterium to be determined or is a sequence complementary to fragments or derivatives of said spacer sequences, said oligonucleotide probe being capable of hybridising to such a spacer sequence and comprising at least ten consecutive nucleotides homologous to such a spacer sequence and/or exhibiting at least 60% homology, preferably exhibiting at least 80% homology with such a spacer sequence.

15. A method according to any of claims 12-14 wherein the bacterium is a pathogenic bacterium selected from the group of Gram negative bacteria of Groups 4 and 5 of Bergeys Determinative Manual of Bacteriology ninth edition, in particular the families Enterobacteriaceae, Pasteurellaceae and Vibrionaceae of Group 5, most specifically the Enterobacteriaceae and the Gram positive bacteria of Group 17.

16. A method according to any of claims 12-15 wherein the bacterium

is a pathogenic bacterium selected from the group of Gram negative bacteria of Bergeys Determinative Manual of Bacteriology ninth edition of the genera Escherichia, Shigella, Salmonella, Klebsiella, Enterobacter, Yersinia, Serratia, Haemophilus, Vibrio, Legionella, Neisseria, Pseudomonas and Bordetella and the group of Gram positive bacterial genera Staphylococcus and Streptococcus as target for the differentiation method.

17. A method for differentiating the type of bacterium in a sample, said bacterium not belonging to the M. tuberculosis complex, said method comprising carrying out the method according to any of claims 12-16, followed by comparison of the hybridisation pattern obtained with a reference.

18. A method according to claim 17, wherein the reference is the hybridisation pattern obtained with one or more known strains of the bacterium to be determined in analogous manner.

19. A method according to claim 17 or 18 wherein the reference is a source providing a list of spacer sequences and sources thereof, such as a data bank.

20. A pair of primers wherein both primers comprise oligonucleotide sequences of at least 7 oligonucleotides and are sufficiently complementary to a part of the Direct Repeat sequence of the microorganism E. coli for hybridisation to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction and wherein sufficiently complementary means said oligonucleotide sequence comprises at least seven consecutive nucleotides homologous to such a Direct Repeat sequence in particular a Sequence from Table II and/or exhibits at least 60% homology, preferably at least 80% homology, most preferably more than 90% homology with the corresponding part of the Direct Repeat sequence.

21. Primer pair according to claim 21, comprising one primer DRa capable of elongation in the 5' Direction and the other primer DRb capable of elongation in the 3' Direction with DRa being complementary to

a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary, the Direct Repeat being present in the Direct Region of E. coli.

22. A pair of primers wherein both primers comprise oligonucleotide sequences of at least 7 oligonucleotides and are sufficiently complementary to a part of the Direct Repeat sequence of the microorganism S. typhimurium for hybridisation to occur and subsequently elongation of the hybridised primer to take place, said primers being such that elongation in the amplification reaction occurs for one primer in the 5' Direction and for the other primer in the 3' Direction and wherein sufficiently complementary means said oligonucleotide sequence comprises at least seven consecutive nucleotides homologous to such a Direct Repeat sequence in particular the E. coli Sequence of Table II and/or exhibits at least 60% homology, preferably at least 80% homology, most preferably more than 90% homology with the corresponding part of the Direct Repeat sequence.

23. Primer pair according to claim 22, comprising one primer DRa capable of elongation in the 5' Direction and the other primer DRb capable of elongation in the 3' Direction with DRa being complementary to a sequence of the Direct Repeat located to the 5' side of the sequence of the Direct Repeat to which DRb is complementary, the Direct Repeat being present in the Direct Region of S. typhimurium.

24. Kit for carrying out a method according to any of claims 1-19, comprising a primer pair according to any of claims 20-23 and optionally an oligonucleotide probe or a carrier, said carrier comprising at least 1 oligonucleotide probe specific for a spacer region of a bacterium to be determined said bacterium not belonging to M. tuberculosis complex, preferably the oligonucleotide probe as defined, said oligonucleotide probe being an oligonucleotide probe of at least 10 nucleotides, preferably more than 12 nucleotides, in particular comprising between 12 to 40 nucleotides, said probe being sufficiently homologous to any of the spacer sequences or to fragments or derivatives of such spacer sequences to hybridise to such a spacer sequence, said oligonucleotide probe comprising at least ten consecutive nucleotides homologous to such a spacer sequence and/or exhibiting at least 60% homology, preferably exhibiting at least 80% homology, most preferably exhibiting more than

90% homology with the corresponding part of the spacer sequence.

25. Kit according to claim 24 further comprising a data carrier with required reference patterns of the bacterial strain to be determined.

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Fig 1

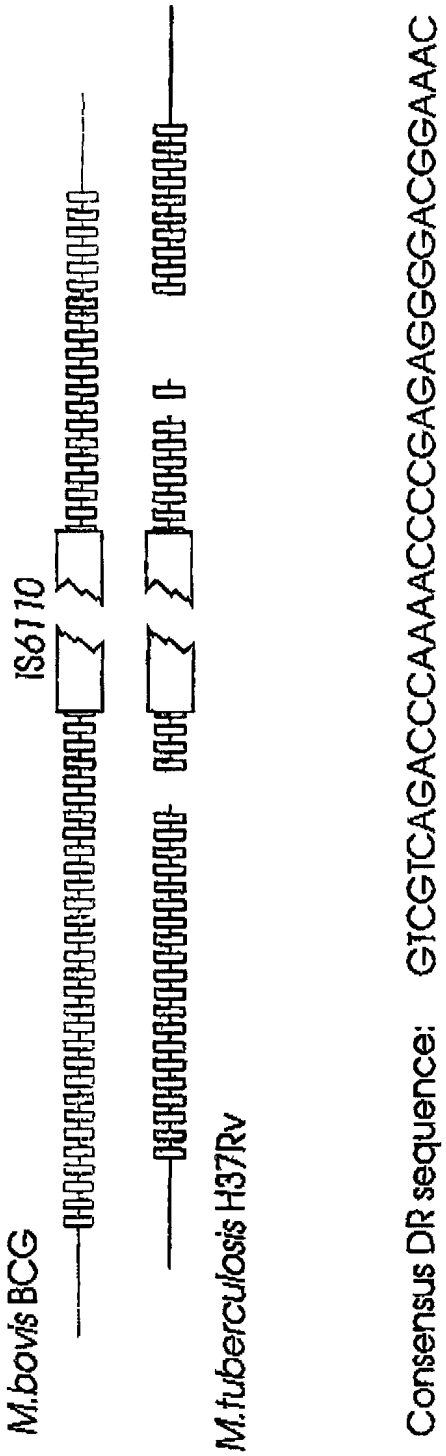


Fig 2

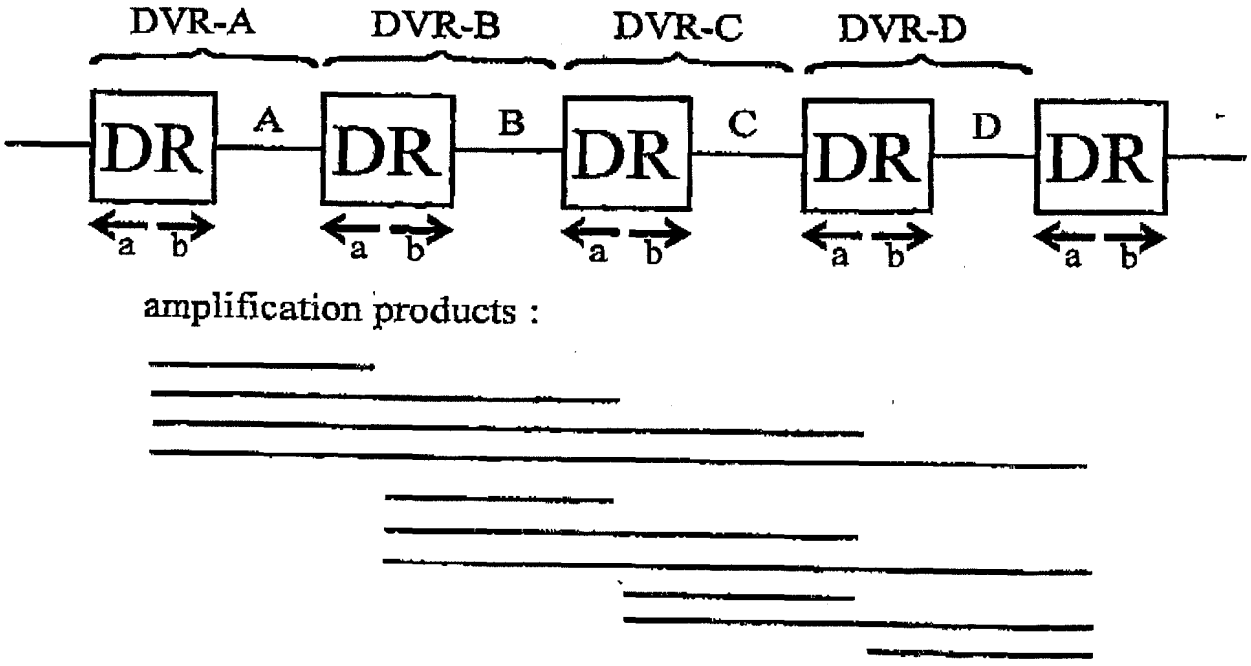
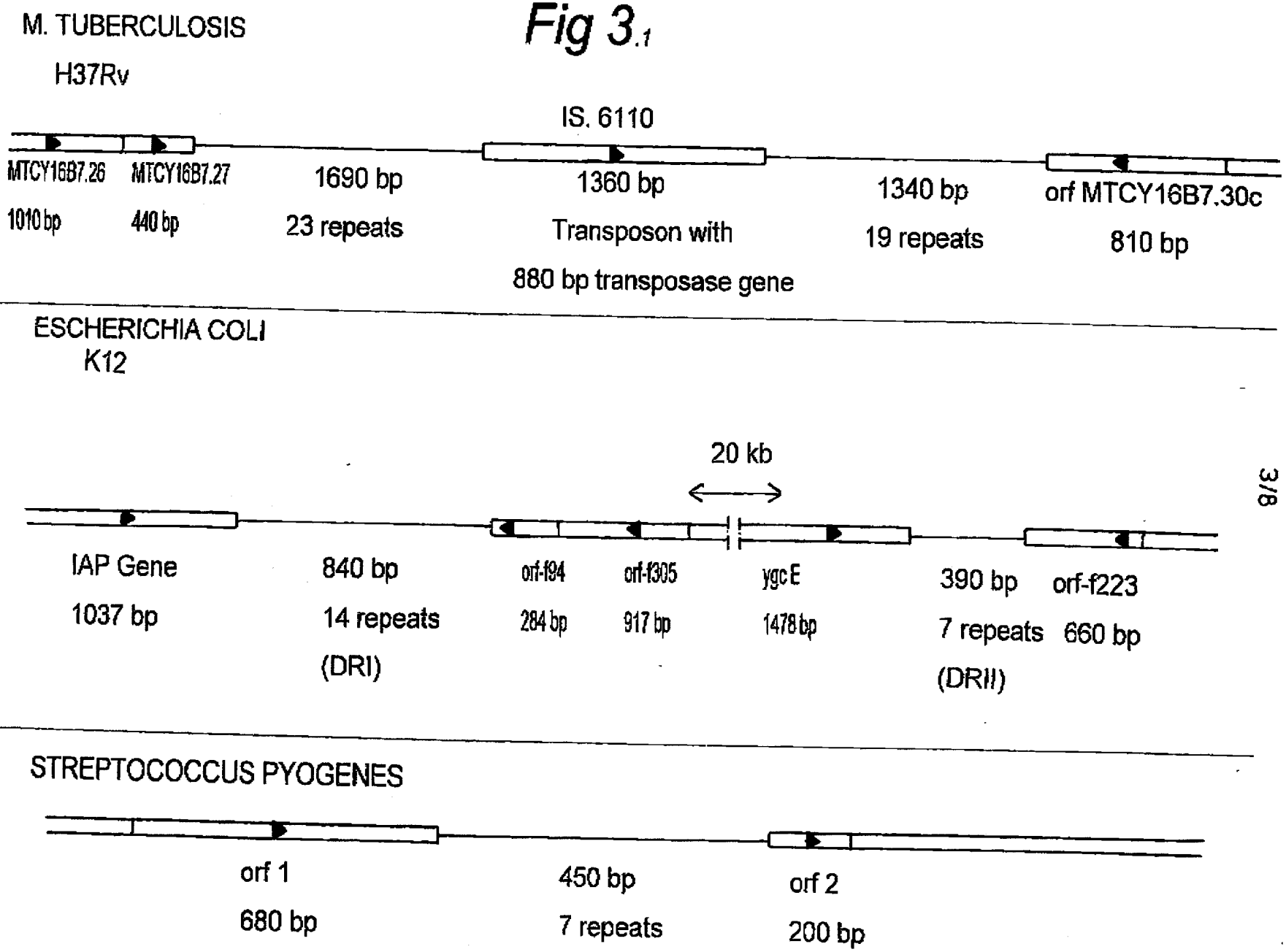


Fig 3.1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K12
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IAP Gene
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840 bp
14 repeats
(DRI)
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284 bp
orf-f305
917 bp
ygc E
1478 bp
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(DRII)
orf-f223
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STREPTOCOCCUS PYOGENES
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200 bp

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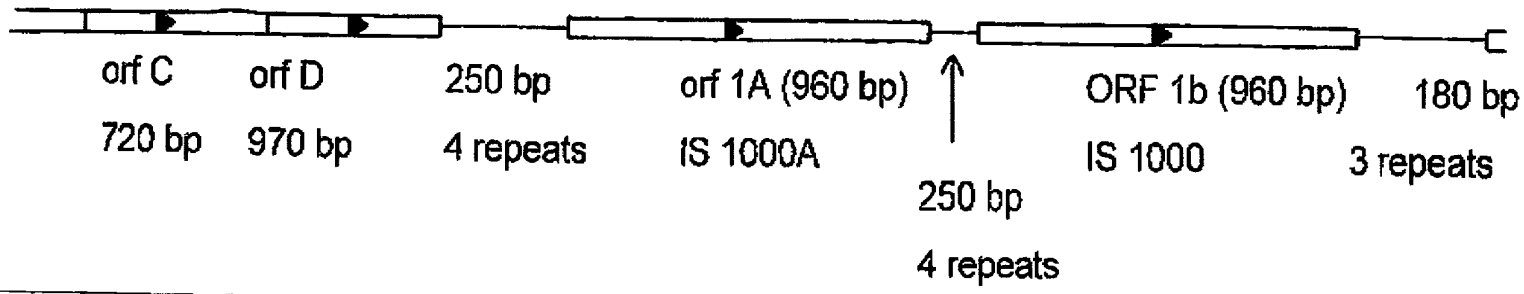
Fig 3.2



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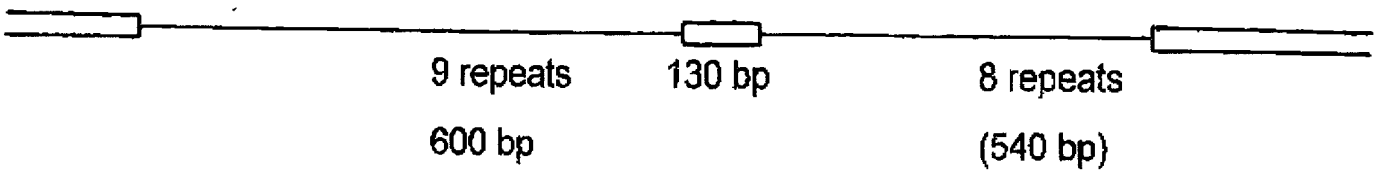

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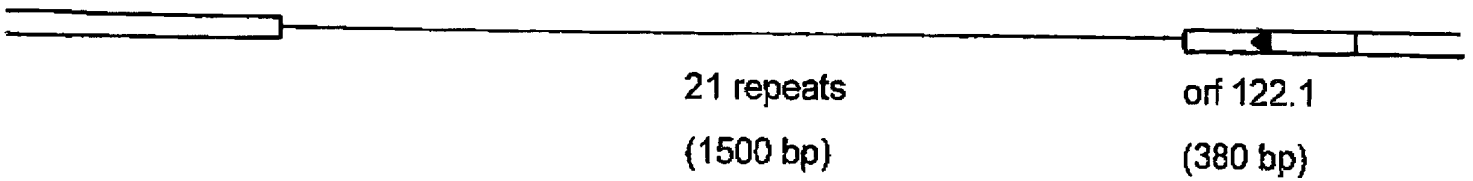
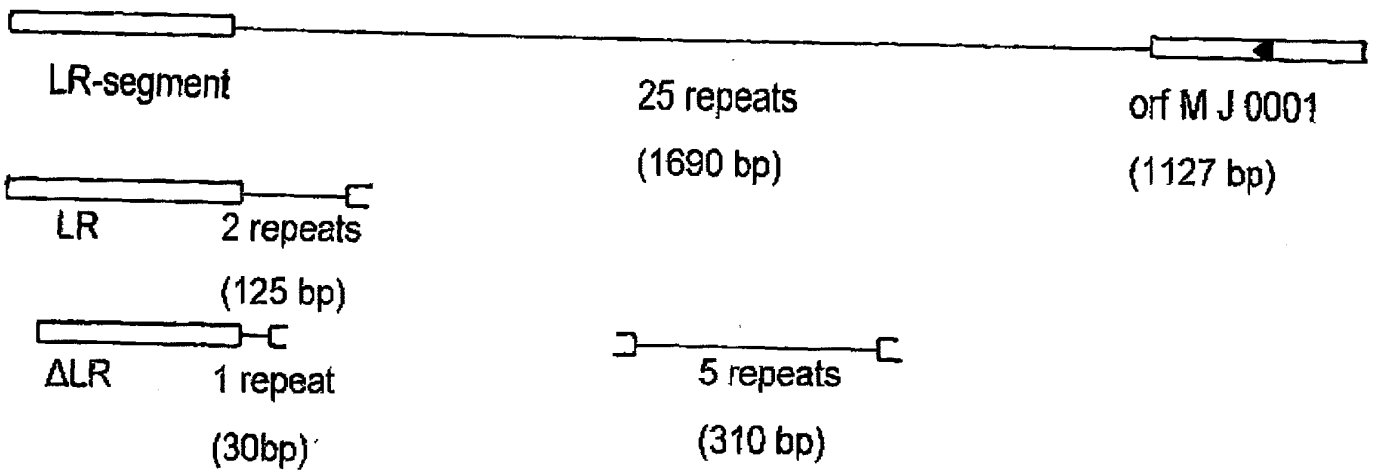


METHANOCOCCUS JANNASCHII

Fig 3.3



METHANOBACTERIUM THERMOAUTOTROPHICUM

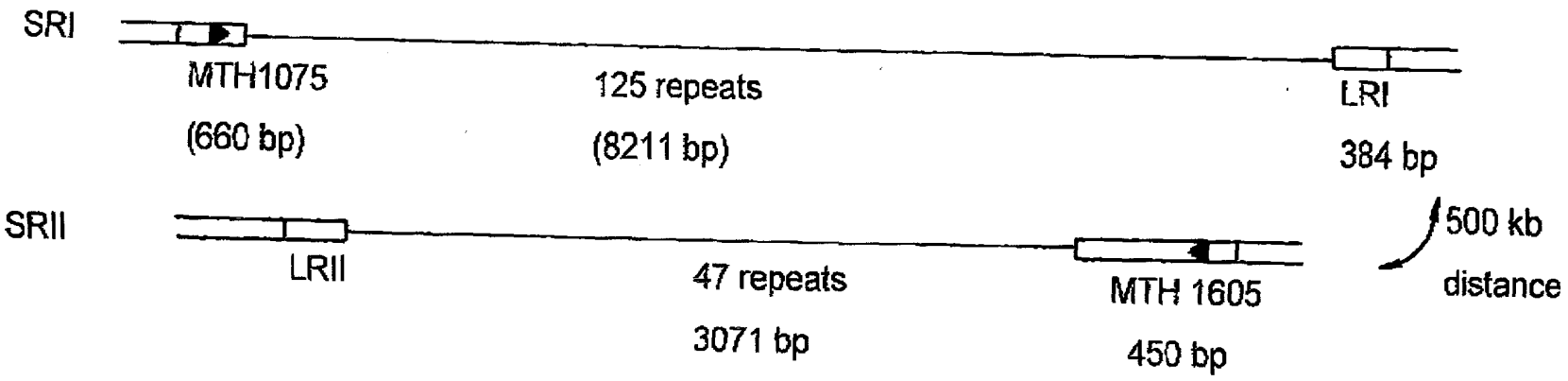
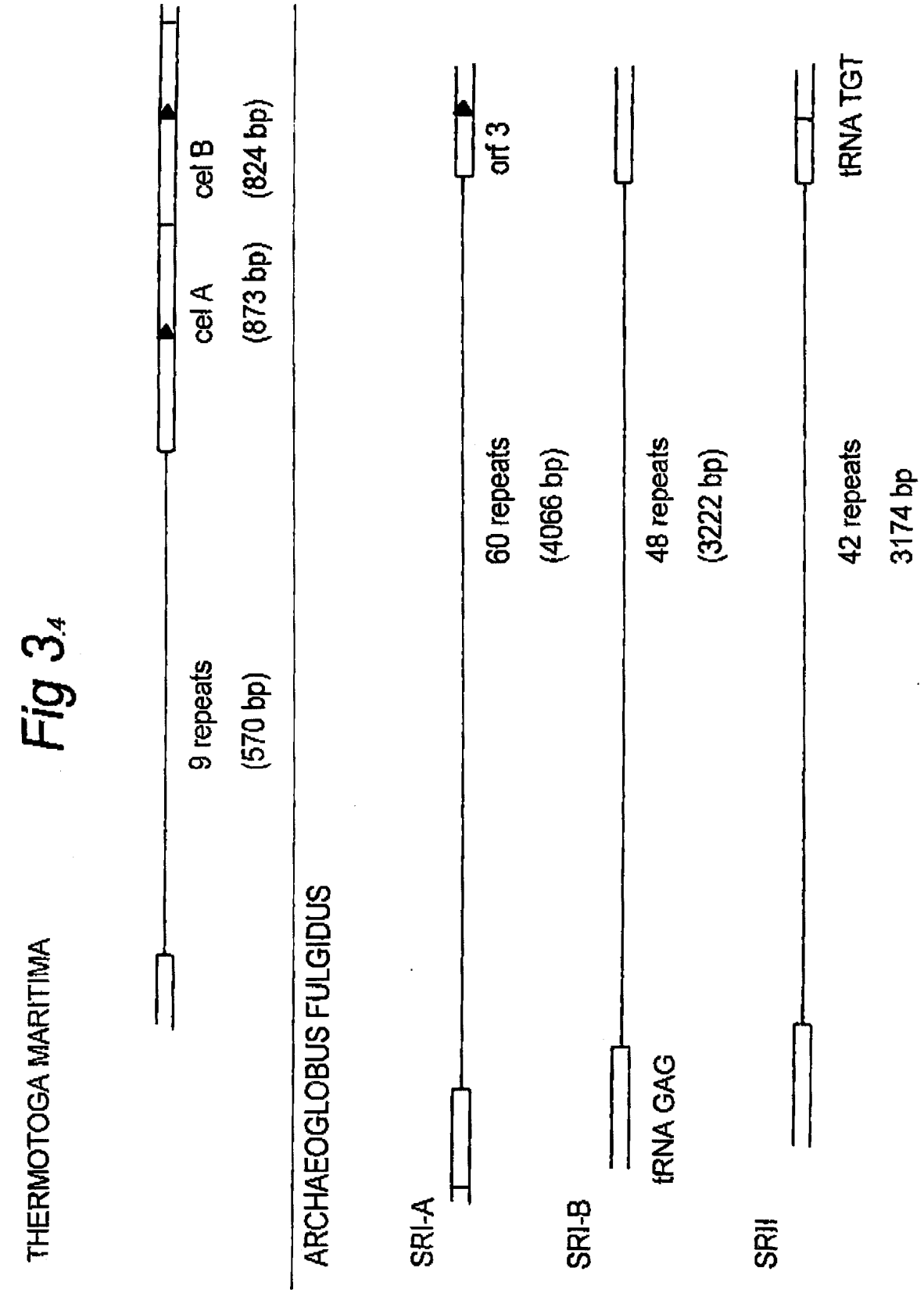


Fig 3.4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cel A
(873 bp)
cel B
(824 bp)
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SRI-A
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(4066 bp)
orf 3
SRI-B
48 repeats
(3222 bp)
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tRNA TGT

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Fig 4

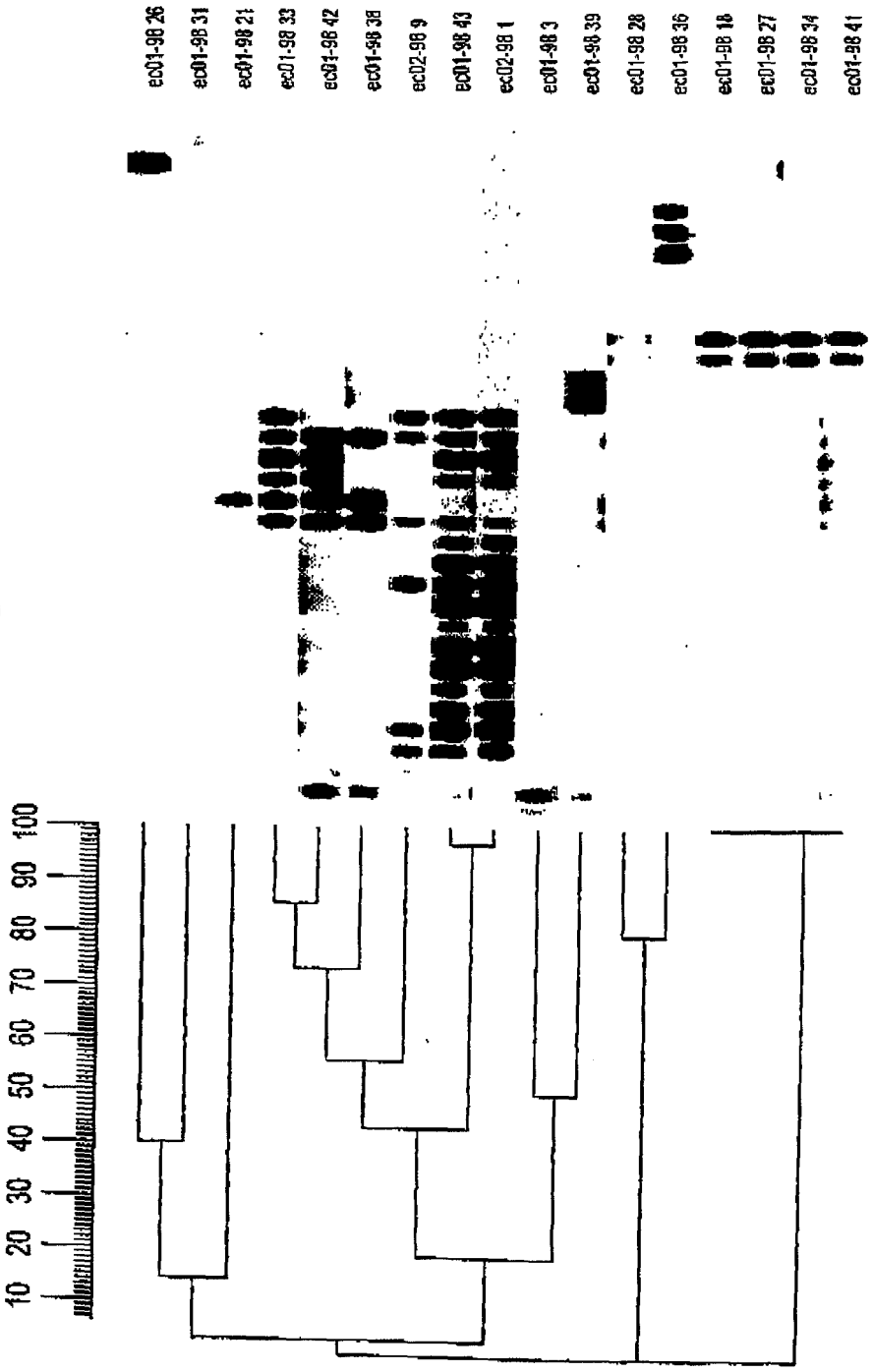

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Fig 5

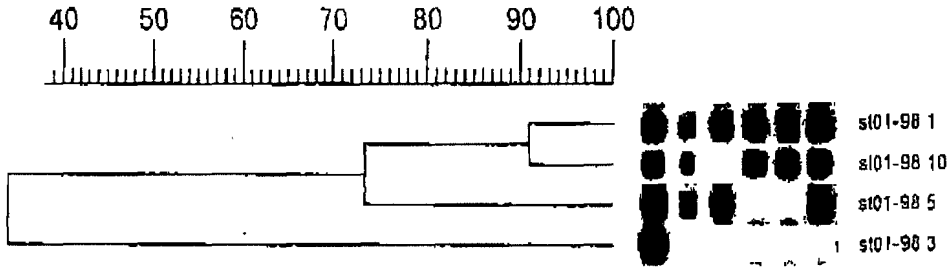

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Fig. 6

SEQ ID No. 1: GTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAAC

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SEQ ID No. 3: CGGTTTATCCCCGCTGGCGCGGGGAACTC

SEQ ID No. 4: CGGTTTATCCCCGCTGGCGCGGGGAACTC

SEQ ID No. 5: CGGTTTATCCCCGCTGGCGCGGGGAACTC

SEQ ID No. 6: CGGTTTATCCCCGCTGGCGCGGGGAACTC

SEQ ID No. 7: CGGTTTATCCCCGCTGGCGCGGGGAACTC

SEQ ID No. 8: CGGTTTATCCCCGCTGGCGCGGATACAC

SEQ ID No. 9: GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACT

SEQ ID No. 10: AATCCCCTTACGGGGCTCAATCCCTTGCAA

SEQ ID No. 11: GTTTCAATACTTCCTTAGAGGTATGGAAAC

SEQ ID No. 12: GTTTTAACTAACAAAAATCCCTATCAGGGATTGAAAC

SEQ ID No. 13: GTTTAAACTTTATAAAATCCCTTTTAGGGATTGAAAC

SEQ ID No. 14: GTTACAGACGAACCCTAGTTGGGTTGAAGC

SEQ ID No. 15: AATTAAAATCAGACCGTTTCGGAATGGAAA

SEQ ID No. 16: ATTTCAATCCCATTTTGGTCTGATTTTAAC

~~SEQ ID No. 17~~: GTTAAAATCAGACCAAAATGGGATTGAAAT

SEQ ID No. ~~18~~ 17: CTTTCAATCCCATTTTGGTCTGATTTCAAC

AMENDED SHEET

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 98/00186

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 31569 A (NEDERLANDEN STAAT ;EMBDEN JOHANNES DIRK ANTHONIE (NL); SCHOULS LEE) 23 November 1995 cited in the application see the whole document	1,3-6, 10-14, 17-25
X	KAMERBEEK J ET AL.: "Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 35, no. 4, 1997, pages 907-914, XP002091620 cited in the application see the whole document	1,3-6, 10-14, 17-25

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 1999	16/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Knehr, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 98/00186

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	GROENEN P M ET AL.: "Nature of DNA polymorphism in the direct repeat cluster of Mycobacterium tuberculosis; Application for strain differentiation by a novel typing method" MOLECULAR MICROBIOLOGY, vol. 10, no. 5, 1993, pages 1057-1065, XP002091621 cited in the application see the whole document	1,3-6, 10-14, 17-25
X	SOOLINGEN VAN D ET AL: "USE OF VARIOUS GENETIC MARKERS IN DIFFERENTIATION OF MYCOBACTERIUM BOVIS STRAINS FROM ANIMALS AND HUMANS AND FOR STUDYING EPIDEMIOLOGY OF BOVINE TUBERCULOSIS" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 32, no. 10, October 1994, pages 2425-2433, XP000647581	17-19
Y	see the whole document	8-12,15, 16
X	US 5 691 136 A (KOEUTH THEARITH ET AL) 25 November 1997	1,8-12, 15-19
Y	see the whole document	20-22
Y	KLENK H-P ET AL.: "The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon Archaeoglobus fulgidus" NATURE, vol. 390, 1997, pages 364-370, XP002091622 cited in the application see abstract; table 1	20-22
Y	MOJICA F J M ET AL.: "Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferx volcanii and could be involved in replicon partitioning" MOLECULAR MICROBIOLOGY, vol. 17, no. 1, 1995, pages 85-93, XP002091623 cited in the application see abstract see page 85, column 2, paragraph 1 - page 87, column 1, paragraph 2; figure 1	1,3-6

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INTERNATIONAL SEARCH REPORT

Intr ional Application No
PCT/NL 98/00186

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
Y	LIEBL W ET AL.: "Analysis of a Thermotoga maritima DNA fragment encoding two similar thermostable cellulases, CelA and CelB, and characterization of the recombinant enzymes" MICROBIOLOGY, vol. 142, 1996, pages 2533-2542, XP002091624 see abstract see page 2536, column 1, paragraph 3 - page 2537, column 1, paragraph 1; figures 3,4	1,3-6
Y	SHANGKUAN Y-H ET AL.: "Diversity of DNA sequences among Vibrio cholerae O1 and non-O1 isolates detected by whole-cell repetitive element sequence-based polymerase chain reaction" JOURNAL OF APPLIED MICROBIOLOGY, vol. 82, no. 3, 1997, pages 335-344, XP002091625 see the whole document	1,3-6, 8-12,15, 16
A	SOOLINGEN VAN D ET AL: "COMPARISON OF VARIOUS REPETITIVE DNA ELEMENTS AS GENETIC MARKERS FOR STRAIN DIFFERENTIATION AND EPIDEMIOLOGY OF MYCOBACTERIUM TUBERCULOSIS" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 31, no. 8, August 1993, pages 1987-1995, XP000647582 cited in the application	
A	VERSALOVIC J ET AL.: "Distribution of repetitive DNA sequences in eubacteria and application to fingerpriting of bacterial genomes" NUCLEIC ACIDS RESEARCH, vol. 19, no. 24, 1991, pages 6823-6831, XP002091626 see the whole document	

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 98/00186

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2,7
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Subclaims 2 and 7 revealed to be not searchable since they are unclear and lack clarity (claim 2) and comprise no technical features suitable to perform any search (claim 7).

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No
PCT/NL 98/00186

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9531569 A	23-11-1995	AU 690118 B	23-04-1998
		AU 6858294 A	05-12-1995
		EP 0760005 A	05-03-1997
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